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Immortalized Chromaffin Cells Disimmortalized with Cre/lox Site-Directed Recombination for Use in Cell Therapy for Pain after Partial Nerve Injury

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To prepare immortalized adrenal chromaffin cells for eventual clinical use, the immortalizing oncogene must be removed. We have utilized a Cre-mediated excision of a loxP-flanked Tag sequence to test whether immortalized chromaffin cells could be disimmortalized by this method. Cultures of embryonic rat adrenal cells were immortalized with the tsA-TN retroviral vector encoding the loxP-flanked temperature-sensitive allele of SV40 large T antigen (tsA-TN) and a positive/negative *neo/HSV-TK* sequence for selection with either G418 or gancyclovir, respectively. These cells were then infected with the 1710-CrePR1 bicistronic retroviral vector coding for a form of Cre modulatable by the synthetic steroid RU486. These immortalized loxTsTag/CrePR1/RAD cells expressed immunoreactivities (ir) for all the catecholamine enzymes: tyrosine hydroxylase (TH), dopamine β-hydroxylase (DβH), and phenylethanolamine-*N*-methyltransferase (PNMT). After initial incubation at 37°C with RU486 for 3 days, followed by the addition of gancyclovir for 7 days, Tag-ir was not detectable in most of the surviving chromaffin cells, compared to 100% expression in immortalized loxTsTag/CreR1/RAD cells not treated with RU486 and gancyclovir. The expression of TH, DβH, and PNMT was increased after disimmortalization and the ability of disimmortalized cells to synthesize norepinephrine was also significantly increased compared to immortalized cells. When both types of chromaffin cells were transplanted in a model of neuropathic pain and partial nerve injury, both cell grafts were equally able to reverse the behavioral hypersensitivity induced by the injury. The use of Cre/lox site-directed disimmortalization of chromaffin cells that are able to deliver neuroactive molecules offers a novel approach to cell therapy. © 2002 Elsevier Science (USA)

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INTRODUCTION

Transplanted primary adrenal chromaffin cells have been used for delivery of therapeutic molecules for a variety of therapeutic indications (56). However a serious limitation is the necessity of harvesting fresh cells from donors, requiring safety screening for each batch of cells and a resultant mixture of cell types which is incompletely characterized and nonhomogeneous. The generation of chromaffin cell lines, utilizing the temperature-sensitive allele of SV40 large T antigen (tsTag) has recently been reported (15), and such cells are able to reverse neuropathic pain after transplant in the spinal subarachnoid space (19). Even with 100% disappearance of Tag in the grafts within a few weeks after transplant, oncogene expression *in vivo* remains a potential possibility and such cells would not be an appropriate strategy for safe clinical use in humans.

Studies exploiting site-specific DNA recombination and Cre/lox excision have suggested that cells can be targeted *in vitro* (25, 50, 51) and *in vivo* (28, 49) for removal of deleterious genes, including Tag (43). Reversible immortalization with Tag and Cre/lox technology was first reported with human fibroblasts by Westerman and Leblouch (61) and more recently with human myogenic cells and hepatocytes (4, 30, 37, 38). In these latter studies, Cre was introduced by transfection or infection, inefficient methods that may lead to a lack of disimmortalization and the loss, through the subsequent selection of disimmortalized cells, of a significant part of the population. Moreover, *in vivo* excision is not possible. Use of a vector that allows a silent, but inducible, form of Cre is preferred for the timed excision of the oncogene.

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A number of chimeric Cre-containing fusion proteins, especially fusions with the ligand-binding domains of steroid receptors, have been created to utilize the binding by synthetic ligands to activate Cre (42). CrePR1 is a fusion protein (36) consisting of the fusion of Cre and the ligand-binding domain of a mutant human progesterone receptor (hPRB891). Cre activity in the cells is activated by the binding of the steroid RU486, which in turn induces the translocation of CrePR1 to the nucleus where the Cre is active to excise the floxed sequences. The requirement for RU486 and the use of a mutated steroid receptor for disimmortalization would assure that if nondisimmortalized cells were transplanted, Cre would not be activated by circulating endogenous progesterone, a strategy used for inducible recombination with *in vivo* CNS studies (35).

Here we describe and expand our recent preliminary reports (16) for immortalization/disimmortalization of chromaffin cells using the tsTag and modulatable Cre-mediated excision of the tsTag sequence. Preliminary data describing the use of these grafted and disimmortalized cells for the relief of neuropathic pain has also been recently reported (17), suggesting that chromaffin cells disimmortalized by such Cre-mediated excision before transplant *in vitro* are still able to have a antinociceptive function.

MATERIALS AND METHODS

Animals. Sprague–Dawley (for establishment of chromaffin lines) and Wistar–Furth (for behavioral studies) rats were obtained from Harlan Labs. All surgical interventions and pre- and postsurgical animal care and euthanasia were in accordance with the Laboratory Animal Welfare Act, *Guide for the Care and Use of Laboratory Animals* (NIH, DHEW Pub. No. 78-23, Revised, 1978) and guidelines provided by the Animal Care and Use Committee of the University of Miami, Miami, Florida.

Vector constructions and establishment of retroviral producer lines. Plasmid construction was done using standard procedures (2). The temperature-sensitive T antigen of SV40 (tsA-Tag, kindly donated by J. Feunteun) with a *LoxP* sequence flanking its 5′ end was inserted downstream of the IRES sequence contained in the LNL6-based p1704 retroviral backbone (kindly donated by J. E. Majors). The resulting retroviral vector was designated as p1704/tsA. In a second step, the hybrid resistance gene obtained through the fusion of the genes for the herpes simplex virus thymidine kinase (*HSV TK*) and the bacterial neomycin phosphotransferase (*neo*) (from the plasmid pTNFUS69 kindly donated by R. Kucherlapati) with an upstream *LoxP* sequence was inserted upstream of the IRES sequence in p1704/tsA to obtain the plasmid ptsA-TN(LoxP). p1710(CrePR1) was obtained by inserting, into the p1704 backbone, the CrePR1 fusion gene (obtained through the fusion of Cre recombinase and the ligand-binding domain of the mutant human progesterone receptor HBR891, a kind gift of Dr. F. Tronche) upstream of the IRES sequence and a hygromycin-B-phosphotransferase gene downstream.

ψ2 packaging cells were grown in DMEM containing 10% fetal calf serum and 100 U/ml penicillin and streptomycin (pen-strep) at 37°C in 5% CO_2/95% air. For the production of the retroviral vector, cells were plated onto 60-mm culture dishes and transfected by lipofection using 20 μl Lipofectamine (Gibco BRL) and 5 μg plasmid DNA. Selection was begun 2 days later using 600 μg/ml G418 (Gibco BRL; for production of tsA-TN(LoxP) producer line) or 400 μg/ml hygromycin-B (Boehringer; for the production of the p1710 (CrePR1) producer line) and continued for 2 weeks. Clones were obtained from the surviving cells by plating onto 96-well plates by limiting dilution and expanded. Test for the presence of helper virus was performed using the proviral rescue assay (2) and was found to be negative.

Primary culture and immortalization of dissociated rat chromaffin cultures. Methods to culture chromaffin cells from rat embryonic adrenal tissue has been previously described (24). Briefly, primary cultures of rat chromaffin cells were established from Sprague–Dawley rat embryos on fetal day 17 (E17). The whole adrenal was dissected out and pooled in Mg^{2+}–Ca^{2+}-free Hanks' balanced salt solution with pen-strep (CMF-HBSS) on ice. The tissue was incubated at 37°C for 15 min in 1 ml DNase I (1×)/0.125% trypsin/CMF-HBSS. After trituration through increasingly finer bore cotton-plugged glass pipettes, the settled tissue was rinsed ×1 with heat-inactivated horse serum (HIHS) to inactivate the trypsin. Tissue was settled, the top layer of cells diluted to 2 ml with the growth media (DMEM/RMPI 1640 (1:1, v/v)/20% HIHS/pen-strep/glutamine (2 mM). Cells ($0.5–1 \times 10^4$), counted by trypan blue exclusion, were plated on a six-well plate (Costar). After 6–12 h, at 37°C, 5% CO_2, medium was changed to remove dead cells and cultures were grown overnight before infection with immortalizing viruses.

Cultures were incubated overnight with the conditioned medium from the competent retroviral producer line ψ2-tsA-TN(LoxP), in the presence of polybrene 4 μg/ml (Sigma). Cultures were rinsed with medium to remove virus and allowed to rest for 2 days at 33°C, before treating cultures with the addition of 250 μg/ml of the selection antibiotic G418 (geneticin; Gibco) in selection medium (DMEM/F12, D/F medium; GIBCO) plus 10% fetal bovine serum (FBS)/pen-strep). When cells were visible after 3–5 days, the FBS was increased to 20% to increase the rate of proliferation, the G418 was decreased to 125 μg/ml for maintenance, and cultures were purified by differential plating, to reduce

the fibroblast population, before subcloning in 100-mm TC dishes (~1000 cells/plate) to isolate individual colonies of chromaffin cells with subcloning rings. Good survival of chromaffin clones required that some fibroblasts were carried through each subcloning, and each final chromaffin line contains a small number of adherent fibroblasts which must be removed by differential plating (15) before use.

These immortalized chromaffin cells were further subcloned after overnight infection at 37°C with the conditioned media from the competent retroviral producer line ψ2-1710(CrePR1), in the presence of 4 μg/ml polybrene. Cultures were rinsed with media to remove virus and allowed to rest for 2 days at 33°C, before treating cultures with the addition of 250 μg/ml of the selection antibiotic hygromycin (Boehringer-Mannheim). As with initial immortalization, hygromycin concentration was decreased to 125 μg/ml for maintenance, and cultures were purified by differential plating, to reduce the fibroblast population, before subcloning in 100-mm TC dishes with subcloning rings to isolate individual colonies of CrePR1-expressing chromaffin cells. A rat chromaffin cell line was isolated and used for the studies here described: the lox/tsTagRAD/CrePR1.

After expansion with proliferation, chromaffin cultures were suspended in Cellvation (Celox Labs) freezing medium, according to the manufacturer's directions, for permanent liquid N2 cell storage. Various passages of these chromaffin cell lines have been frozen, warmed, reconstituted, and expanded for more than 2 years, although passage numbers of about 10–15 are most commonly used for the *in vitro* characterization and transplant. No passage number greater than 45 has been used for experiments, but no detectible difference in growth or phenotype characteristics has been observed with these later passage numbers.

Cell culture, excision of TsTag, and transplantation of rat chromaffin clones. For differentiation, the immortalized rat chromaffin cell line was grown at nonpermissive temperature (39°C) in B16 base medium (5, 48) containing 1% (w/v) bovine serum albumin (BSA; (Boehringer-Mannheim), TCM proprietary serum-free replacement ingredients (1× concentration; Celox Labs) and pen-strep, for the number of days indicated in each experiment. Differentiation was continued in differentiation media with the addition of 5 μg/ml dexamethasone (DEX) (15) and 500 μM tetrahydrobiopterin (BH4), the cofactor for catecholamine synthesis (1).

The methods for transplantation of similarly immortalized rat chromaffin cells into the subarachnoid space of the lumbar spinal cord after nerve injury for pain relief has been described previously (19). The chromaffin cells were proliferated to near confluence at permissive temperature (33°C), in DMEM/Ham's F12 (DF) media/10% FBS/125 μg/ml G418 as described previously (15), before switching for 10 days to the differentiation media and 37°C. Separate cultures of cells were left untreated or incubated for the first 3 days in the presence of 1 μM RU486, with 40 μg/ml gancyclovir added the last 7 days of treatment, at 37°C. Both cultures were differentially plated to remove fibroblasts after excision and settled overnight on six-well plates. Immediately before transplantation, cells were gently dissociated with sterile 0.5 mM EDTA/DPBS, counted by trypan blue exclusion to ensure equal numbers of viable chromaffin cells were transplanted, pelleted by centrifugation, and suspended in a concentration of in $10^6/\mu$l of CMF-HBSS. Adult female (180 g) Wistar–Furth rats were used for transplantation. Following a partial laminectomy and a small puncture of the dura, chromaffin cells (106) were injected into the subarachnoid space of the lumbar dorsal spinal cord, by a dorsal/caudal entry into the dural puncture a few millimeters with a small length of polyethylene (PE-10) tubing containing the cells, at spinal segment L1, 1 week after the CCI. For both CCI and transplantation surgeries, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine, 0.65 ml/kg. Animals were allowed to recover at 37°C for 12 h, after which time they were returned to the animal care facility, and housed one per cage with rat chow and water ad lib on a 12/12-h light/dark cycle.

Immunocytochemistry: In vitro. For characterization of the chromaffin cell lines using immunocytochemistry, cells were proliferated to near confluence at permissive (33°C) before switching to the differentiation media and 37°C. As above, separate cultures of cells were left untreated or incubated for 3 days in the presence of 1 μM RU486, with 40μg/ml gancyclovir added to the differentiation media the last 7 days of treatment, at 37°C. Both cultures were differentially plated after excision to remove fibroblasts and settled overnight on eight-well plastic slides. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 min, rinsed with phosphate-buffered saline (PBS), and nonspecific background blocked with the nonimmune serum, for a few hours at the room temperature. Cells were reacted with primary antibodies diluted in PBS/0.4%Triton X-100 (TX)/5% normal goat serum (NGS) for 18–48 h at 4°C, followed by several rinses and incubation in secondary fluorescent antibodies (1–2 h at room temperature). After reactions were completed, slides were rinsed and coverslipped using antifade mounting medium. Cells that were only stained for Tag were double labeled with DAPI to identify and count viable cells. Negative controls were done in the absence of primary antibody and positive controls on noninfected cells (primary chromaffin cells). Primary antibodies were monoclonal anti-Tag (hybridoma PB101 supernatant, clone 412,

American Type Culture Collection (Rockville, MD), used at 1:1–1:5 dilution); polyclonal anti-TH (1:300; Chemicon, Temecula, CA); polyclonal anti-DH (1:300; Inscstar, Stillwater, MN); and polyclonal anti-PNMT (1:300; Inscstar, Stillwater, MN). Secondary antibody reporters were goat anti-mouse or goat anti-rabbit IgG Alexa green or Alexa red from Molecular Probes (Eugene, OR). After reactions were completed, slides were coverslipped using antifade mounting medium, Vectashield from Vector Laboratories (Burlingame, CA). Cell preparations were scanned with Zeiss inverted confocal microscope (Axiovert 100M), using argon LASOSLGK 7812 ML4/LGN 7812:458-, 477-, 488-, 514-nm, 30-nW laser, furnished with software package LSM 510. Images were collected at the 488-nm wavelength and scanned at 200 dpi into TIF format and images collected in Adobe Photoshop.

Quantitation of immunoreactive Tag (Tag-ir) after treatment with RU486 and gancyclovir. To determine the efficiency of RU486-induced excision of the tsTag sequence *in vitro,* cells were proliferated to near confluence at permissive (33°C) before switching to the differentiation media and 37°C. As above, separate cultures of cells were left untreated or incubated for 10 days in the presence of RU486 alone, 10 days in the presence of gancyclovir alone, or 3 days in the presence of 1 μM RU486 with 40 μg/ml gancyclovir added to the differentiation media the last 7 days of treatment, at 37°C. All resultant cultures were differentially plated to remove fibroblasts and settled overnight on eight-well plastic slides before fixation and staining for cells which colocalize TH and Tag. Representative quantitative data was used from three separate experiments to derive the data. Ten random fields of cells were counted at 40× magnification from each well. Total cell numbers, those stained for DAPI to identify the viable portion of total cells, those stained for TH, and those containing Tag-ir were used to determine how many viable, TH-containing, chromaffin cells still expressed Tag, with the various treatment conditions.

HPLC for catecholamine content in chromaffin cell lines. Chromaffin cell lines were examined for catecholamine neurotransmitters, norepinephrine and epinephrine, under differentiation conditions either with or without treatment with RU486 and gancyclovir (37°C). HPLC methods to examine catecholamines from cells were a modification of those reported previously (10, 11, 15). With both nontreatment and treatment conditions, cell cultures were differentially plated to remove fibroblasts, the chromaffin cells counted by trypan blue exclusion and plated (2×10^6/well) in eight-chamber plastic slides, and the cells settled for 6 h before use for HPLC studies. Cells were washed and then dissolved in 0.05 N PCA, overnight at 4°C for cell lysis, to measure cell content of catecholamines in each well. A 10-μl aliquot of the last was injected onto the HPLC (BAS-200, Analytical System, Inc.) C18 column (150 × 1 mm) with electrochemical detection. The potential used was +750 mV vs Ag/AgCl, with a classic 3-mm glassy carbon electrode. The ranges of sensitivity for the electrode were 50 and 5.0 nA, with a flow rate of 0.10 ml/min. Chromaffin cell lines were examined at the 5.0 nA sensitivity. The mobile phase included 25 mM NaH_2PO_4/50 mM Na citrate/25 μM disodium–EDTA/10 mM diethylamine · HCl/2.2 mM 1-octanesulfonic acid (sodium salt), with the pH adjusted to 3.2 with H_3PO_4. An additional 30 ml of methanol and 22 ml of dimethylacetamide were added to 1 L of this buffer before use. Primary cultures of chromaffin cell were used as positive controls; some samples had stock neurotransmitters added to spike the media and served as internal standards. Ordinarily the norepinephrine appeared at about 3 min; the epinephrine at about 3.5 min.

Chronic constriction injury (CCI). The surgery to produce CCI was first described by Bennett and Xie (3). This model of injury has been used to test the effects of bioengineered cell transplants to relieve the pain-related behaviors (8, 14, 18, 21) and was subsequently used before transplantation of similarly immortalized chromaffin cells (19). Under ketamine/xylazine anesthesia, the right common sciatic nerve was exposed at the level of the middle thigh by blunt dissection through the biceps femoris. Proximal to the nerve's trifurcation, a 5–7 mm of nerve was freed of adhering tissue and four ligatures (4.0 chromic gut) were tied loosely around it with about 1 mm spacing. Care was taken to tie the ligatures so that the diameter of the nerve was barely constricted. The incision was closed in layers and the entire surgery was repeated, minus the ligatures, on the left side to create a sham-operated nerve.

Behavioral testing protocol. One week before CCI and transplants, animals were acclimated and trained for 3 days on all behavioral tests, followed by a baseline measure of the tests described below 1 week before CCI. One week following CCI and every week thereafter animals were retested. Animals to be transplanted were then injected with either the untreated or the RU486/gancyclovir-treated chromaffin cells. A third group of animals received CCI but no transplants and served as the CCI-alone control group. A fourth group of animals received neither CCI or transplants and served as the naive control animals. Another group received sham transplants of an equal number of fibroblasts, but since results are not different from that previously reported (19), and do not differ from the CCI-alone group, the data are not shown here. Behavioral testing, using a blinded rater for all testing, was repeated for all animals once a week for 8 weeks following CCI and transplants. All animals used for be-

havioral testing were sacrificed after 8 weeks of testing.

Thermal hyperalgesia. Methods for testing thermal hyperalgesia with a Hargreaves device have been described elsewhere (3, 29). Animals were placed in a clear plexiglass box on an elevated plexiglass floor. Animals were allowed to acclimate for approximately 5 min. A constant-intensity, radiant heat source was aimed at the midplantar area of the ligated and sham-operated hindpaws. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Five minutes was allowed between stimulations. Five latency measurements for each paw were recorded at weekly sessions before and after CCI and after transplantation. For each of the five latency measurements, the score of the sham-operated paw was subtracted from the ligated paw. From these values, a mean difference score for each session was calculated for each experimental animal and used to determine the effects of the different treatments.

Mechanical allodynia. Mechanical allodynia, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested using a graded series of von Frey hairs (9). Animals were placed in a plexiglass box with an elevated mesh floor. After the animal was acclimated for 5 min, calibrated von Frey hairs with ranges from 0.41 to 8.5g were applied perpendicular to the midplantar area of the ligated and sham-operated hindpaw and depressed slowly until bent. The value, in grams, for the minimal, initial hindpaw withdrawal was recorded for each of five trials. A single trial of stimuli consisted of five applications of a von Frey filament within a 10-s period, to ensure that the response was constant. Each session consisted of five trials, repeated at 3-min intervals on each hindpaw. This minimum value for initial response for the sham-operated paw was subtracted from the score for the ligated paw and all five replicate scores were averaged. From these values, a difference score was calculated and used to determine the effects of the different treatments.

Statistical analysis. Statistical significance of all quantitative data was determined with a multivariate analysis of variance (MANOVA). Comparisons of differences between individual means were tested using the Tukey honest significant differences (HSD) method or the unequal *N* least significant difference (LSD) test. All of the analyses were performed with a commercially available software package (Statistica, Statsoft, 1990). *P* values less than 0.01 were considered statistically significant.

Chemicals. Primary antiserum against the indicated antigens were obtained from the following sources: dopamine β-hydroxylase (DβH), Chemicon; Tag (hybridoma PB101, clone 412), American Type Culture Collection (Rockville, MD); PNMT, Incstar (Stillwater, MN); and TH, Chemicon. Ham's F12 media (DF, 1:1, v/v) and geneticin (G418) were obtained from GIBCO (Grand Island, NY); TCM serum replacement constituent was from Celox Labs and all other powdered media, attachment factors and chemicals for cell culture were purchased from Sigma Chemical (St. Louis, MO). BSA (fraction V, protease-free) was obtained from Boehringer-Mannheim; FBS was from Hyclone (Logan, UT). The RU486 was supplied by Biomol Research Lab, Inc. (Plymouth Meeting, Mass), and the antibiotic gancyclovir was obtained from Roche Laboratories (Nutley, NJ). Cellstripper, Mediatech, Inc. (Herndon, VA.), a proprietary nonenzymatic cell dissociation solution, was used to lift chromaffin cells after differentiation for antibody staining. Vectashield mounting medium (with DAPI) was obtained from Vector Laboratories. Nontissue culture plates for differential plating were obtained from VWR (Plainfield, NJ).

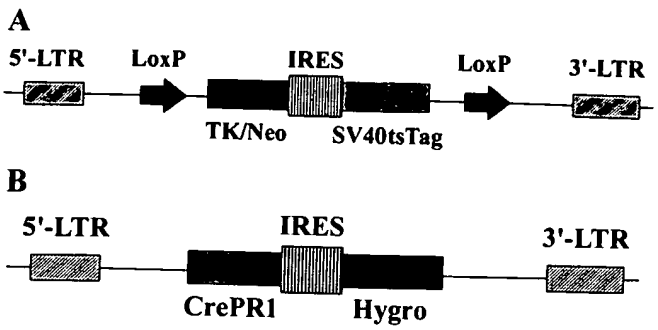

FIG. 1. Scheme of the retroviral vectors used for immortalization and disimmortalization. (A) The tsA-TN(LoxP) construct—LTR, viral long terminal repeat; LoxP, recognition sequence for Cre recombinase; TK/Neo, fusion of the genes for the herpes simplex virus thymidine kinase (HSV TK) and the bacterial aminoglycoside phosphotransferase (neo); IRES, internal ribosomal entry site; tsA-Tag, temperature-sensitive allele of SV40 large T antigen. (B) The1710-(CrePR1) construct—LTR, viral long terminal repeat; CrePR1, fusion of Cre recombinase and the hormone-binding domain of the mutant human progesterone receptor HBR891; IRES, internal ribosomal entry site; Hygro, bacterial hygromycin-B-phosphotransferase.

RESULTS

Vectors used for immortalization and disimmortalization. The immortalizing tsA-TN (LoxP) vector (Fig. 1) fulfills four functions: (i) it immortalizes infected cells, through the expression of the SV40 T oncogene; (ii) allows for the selection of the immortalized cells, by virtue of the aminoglycoside phosphotransferase activity of the TK-neo fusion gene, by the antibiotic geneticin; (iii) the flanking LoxP sequences allow for the excision of most of the provirus when Cre is expressed in the cells; and (iv) cells in which disimmortalization have not occurred can be selected against by the addition of gancyclovir which will be transformed into a toxic drug by the thymidine kinase activity of the

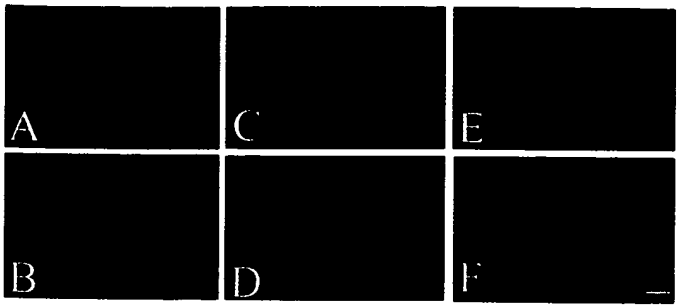

FIG. 2. Disimmortalization of rat chromaffin cells *in vitro*. Equivalent cultures of immortalized chromaffin cells were differentiated for 10 days at 37°C in the absence or presence of RU486 and gancyclovir and stained for TH and Tag. Untreated cells, identified by a low level of TH immunoreactivity (TH-ir) (B), are 100% positive for Tag immunoreactivity (Tag-ir) (A) after 10 days of differentiation. Cells differentiated for 10 days in the presence of RU486 alone continue to be TH-ir (D), but only few cells continue to stain for Tag (C). When similar cells are initially exposed to RU486 for 3 days, followed by 7 days of RU486 plus gancyclovir, the TH-ir cells (F) that survive contain little or no Tag-ir (E). Bar 75 μm.

TK-neo gene. This vector has been validated previously on rat fibroblasts and neuroectodermal cells (30), and the present results show that it can be used to immortalize primary chromaffin cells as well. The use of the 1710 (CrePR1) vector (Fig. 1) allows, through its expression of hygromycin-B-phosphotransferase activity, the establishment of sublines of the immortalized cells that will express the Cre enzyme in a silent form. Indeed, the steroid-binding domain fused to Cre in CrePR1 confines, in the absence of an agonist, the enzyme to the cytoplasm and Cre is then unable to excise the floxed genomic sequences. Although in our hands CrePR1 presents some leakage, maintenance of the cells in G418 removes any spontaneously disimmortalized cells. Activation of Cre activity by RU486 permits, at least theoretically, the disimmortalization of the whole of the population. This procedure offers a more efficient way of disimmortalization than the acute introduction of a Cre expression vector by transfection or infection.

Loss of expression of Tag-ir in rat chromaffin cells after disimmortalization with RU486 and gancyclovir. Qualitative immunohistochemistry for Tag and TH were used to examine the chromaffin cell cultures after 10 days of treatment with RU486 and RU486 followed by gancyclovir to assess what portion of the chromaffin cells continued to contain Tag after RU486 or RU486/gancyclovir treatment, rather than continuing to express high levels of Tag-ir when cells were left untreated. The reduction of Tag-ir with oncogene excision during differentiation in the chromaffin cell cultures is illustrated in Fig. 2. When the chromaffin cells are left untreated for 10 days, 100% of the cells contain Tag-ir (Fig. 2A) and TH (Fig. 2B). After 10 days of differentiation in the presence of RU486 alone, many of the cells cease to label for Tag (Fig. 2C), while continuing to express TH (Fig. 2D). When cells are differentiated in the presence of gancyclovir alone for 10 days, very few or almost no cells survive or stain for the antigens (data not shown). In contrast, when cells are initially treated with RU486, followed by RU486 plus gancyclovir for a total of 10 days, almost no cells contain Tag-ir (Fig. 2E), while they continue to express TH-ir (Fig. 2F). In addition cells are large and rounded.

Quantitation of cell numbers and Tag- and TH-ir after excision with RU486 and gancyclovir. The quantification of the results of treatment of immortalized chromaffin cell cultures with RU486, gancyclovir, or the combination of RU486 and gancyclovir treatment during differentiation *in vitro* is shown in Fig. 3. Treatment of the cells for 10 days with gancyclovir alone resulted in near 100% cell loss, compared to no treatment. Treatment with RU486 alone for 10 days resulted in no significant cell loss and near 60% elimination of Tag-ir from surviving chromaffin cells. In contrast, initial treatment with RU486 for 3 days, followed by RU486 and gancyclovir, resulted in about 50% cell loss, while greater than 82% of surviving cells were Tag free.

Continued expression of tyrosine hydroxylase, dopamine β-hydroxylase and PNMT-ir with differentiation and after tsTag excision in vitro. Immortalized rat chromaffin cells develop a typical rounded chromaffin cell-like morphology. Cells were stained with an antibody specific for TH, DβH, and PNMT (Fig. 4) to qual-

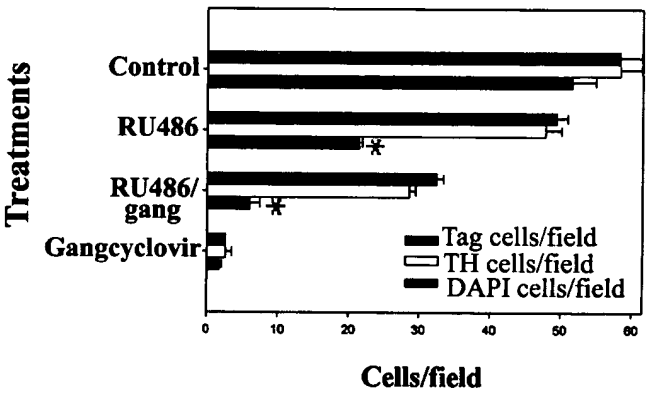

FIG. 3. Quantification of disimmortalization by RU486 and gancyclovir *in vitro*. Immortalized rat chromaffin cells were differentiated at 37°C for 10 days in media alone or with the addition of RU486, gancyclovir, or RU486 for 3 days followed by RU486 plus gancyclovir. Surviving cells were replated to eight-well slides, stained for TH- or Tag, and mounted in DAPI-containing mounting medium to examine viability, and fields of cells were quantified with image analysis software for survival (DAPI staining) and TH- and Tag-antigen immunoreactivity. Data represents the mean ± SEM of 10/fields/well of cells from three independent experiments. ANOVA indicated the existence of statistically significant differences between groups. Asterisks (*) indicate that treatment with RU486 and RU486/gancyclovir decreased the number of Tag-ir surviving cells compared to differentiation in medium alone. $P < 0.01$.

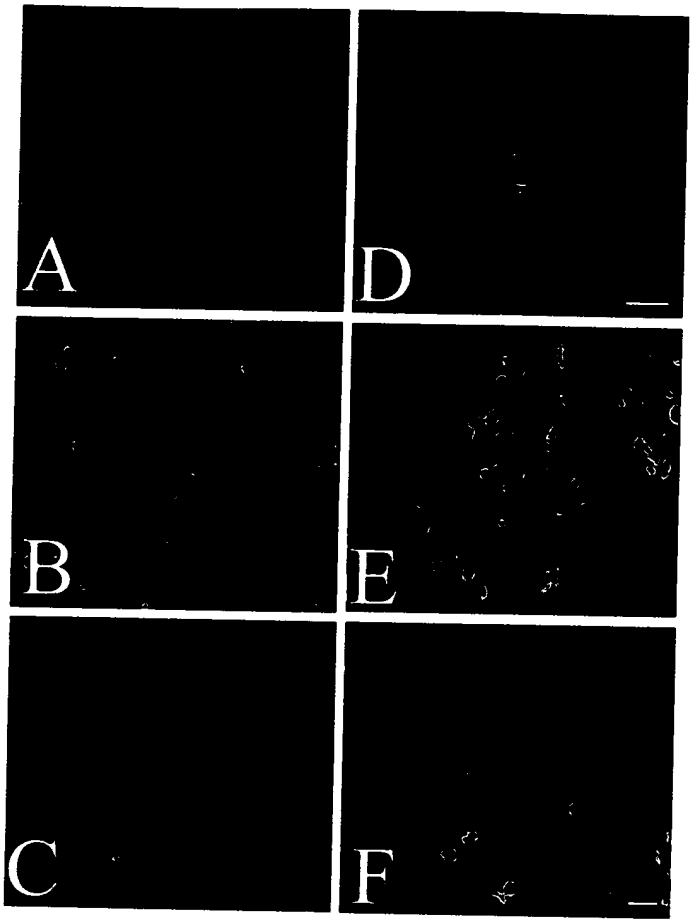

FIG. 4. Catecholamine enzymes in disimmortalized chromaffin cells *in vitro*. Immortalized rat chromaffin cells were differentiated at 37°C for 10 days in medium alone (A–C) or with the addition of RU486 for 3 days followed by RU486 plus gancyclovir (D–F). Surviving cells were replated to eight-well slides and stained for TH (A, D), DβH (B, E), or PNMT (C, F). Bars, 75 μm (A–C, E, F); 150 μm (D).

itatively assess whether the cells expressed the chromaffin catecholamine phenotype after differentiation *in vitro* and with the addition of RU486 and gancyclovir. All three enzymes were expressed, though at low level, by the immortalized cells after 10 days of differentiation (in serum-free media without additions) at 37°C. However, after disimmortalization by RU486 and gancyclovir treatment, the immunoreactivity for each enzyme was visibly increased, compared to untreated chromaffin cultures.

The expression of TH-ir is seen in the chromaffin cells *in vitro* before and after disimmortalization. The immortalized rat chromaffin cells develop a typical rounded chromaffin cell-like morphology and contained barely detectible TH-ir after 10 days of differentiation (in serum-free media without additions) at 37°C (Fig. 4A) and with both RU486 followed by gancyclovir (Fig. 4D) for 10 days at 37°C. However, after RU486 and gancyclovir treatment, the TH-ir was much more visible and brighter in the disimmortalized cells, compared to untreated chromaffin cultures.

The expression of DβH-ir is shown in similar cells and under the same treatment conditions. The immortalized rat chromaffin cells contained easily detectible DβH-ir after 10 days of differentiation (in serum-free media without additions) at 37°C (Fig. 4B) and with both RU486 followed by gancyclovir (Fig. 4E) for 10 days at 37°C. The numbers of DβH-ir cells increased with RU486/gancyclovir treatment.

The expression of PNMT-ir is shown in similar cells and under the same treatment conditions. The immortalized rat chromaffin cells contained detectible PNMT-ir after 10 days of differentiation (in serum-free media without additions) at 37°C (Fig. 4C) and with both RU486 followed by gancyclovir (Fig. 4F) for 10 days at 37°C. Like DβH-ir and TH-ir, both the numbers and the intensity of PNMT-ir apparently increased with RU486/gancyclovir treatment.

HPLC for catecholamine neurotransmitters. Since immortalized rat chromaffin cells express the major synthetic enzymes for these neurotransmitters (15), but do not synthesize detectible catecholamines when the tsTag sequence is present, HPLC methods were again used to examine whether removal of the oncogene induced/allowed neurotransmitter synthesis *in vitro* (Fig. 5). Standard HPLC methods, as described previously for analysis of tsTag-immortalized chromaffin cells, was used (15), which included both appropriate Ca^{2+} and K^+ ions (10, 11) as well as differentiation in the presence of the cofactor BH4 for activity of TH, to examine catecholamine content in these cells. Easily detectible norepinephrine synthesis was observed after excision of the tsTag sequence in surviving chromaffin cells (+), in contrast to similar preparations of untreated and immortalized rat chromaffin cultures (−). No significant epinephrine content was observed in either immortalized or disimmortalized chromaffin cultures.

Thermal hyperalgesia after CCI and chromaffin cell transplants. The measure of sensitivity to noxious heat in animals after nerve injury and transplant of immortalized and disimmortalized rat chromaffin cell transplants is shown in Fig. 6. In control animals without surgery or transplants, no difference was observed between operated and unoperated hindlimbs in the latency of withdrawal over eight weeks (Fig. 6, ○). The range of latency scores was 6.3 to 16.9 s in the right hindpaw and 6.1 to 17.6 s in the left hindpaw at 2 weeks in control animals. After CCI (Fig. 6, ●), a vigorous hypersensitivity to heat was observed in the ligated paw 1 week after CCI and did not recover by 8 weeks. At 2 weeks after CCI, the range of latency scores was 5.0 to 10.9 s in the ligated (right) hindpaw and 6.5 to 18.2 s in the nonligated (left) hindpaw. A

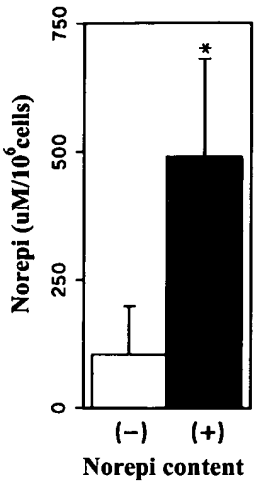

FIG. 5. HPLC for norepinephrine in disimmortalized chromaffin cells. Immortalized rat chromaffin cells were differentiated at 37°C for 10 days in medium alone or with the addition of RU486 for 3 days followed by RU486 plus gancyclovir. With both nontreatment (−) and treatment conditions (+), cell cultures were differentially plated to remove fibroblasts, and the chromaffin cells were counted by trypan blue exclusion and plated (2×10^6/well) in eight-chamber plastic slides, with the cells settled for 6 h before use for HPLC studies. Cells were washed and then dissolved in 0.05 N PCA overnight at 4°C, for cell lysis, to measure cell content of catecholamines in each well. Data represent the mean ± SEM of cells from three independent experiments. ANOVA indicated the existence of statistically significant differences between groups. Asterisks (*) indicate that treatment with RU486/gancyclovir increased the content of norepinephrine in the chromaffin cells compared to differentiation in medium alone. $P < 0.01$.

similar sensitivity was seen in the ligated paw before transplantation of nontreated chromaffin cells (Fig. 6, ▲) at 1 week after CCI.

However, 1 week after transplant of untreated chromaffin cells (2 weeks after CCI), the sensitivity in the ligated hindpaw nearly disappeared. The range of latencies was 6.8 to 16.8 s in the ligated hindpaw and 7.0 to 16.5 s in the nonligated hindpaw at 2 weeks for after transplant of rat chromaffin cells. After transplant of treated chromaffin cells (Fig. 6, ▽), the hypersensitivity observed after CCI in the ligated hindpaw also nearly disappeared and continued for 8 weeks after the CCI. The range of latencies was 7.1 to 16.0 s in the ligated hindpaw and 7.2 to 16.5 s in the nonligated hindpaw at 2 weeks after transplant of untreated chromaffin cells.

Tactile allodynia after CCI and chromaffin cell transplants. The effects of transplants in animals after nerve injury and transplant of immortalized and disimmortalized rat chromaffin cell transplants on mechanical allodynia after CCI are shown in Fig. 7. All animals were examined 1 week before CCI and for 8 weeks after CCI for foot withdrawal in response to stimulation with a graded series of von Frey hairs as described under Materials and Methods. A significant response appeared in the ligated hindpaw 1 week after CCI alone (Fig. 7, ●) that did not resolve during the 8 weeks after surgery. The near maximum nociceptive effect appeared in the ligated paw versus the nonligated paw at 1 week following CCI, with the range of raw latency scores for the ligated paw being 0.025 to 0.675 g, with 1.22 to 3.55 g in the nonligated paw. At the same time period, the scores for the uninjured control animals varied from 1.20 to 3.60 g in either paw (Fig. 7, ○). However, 1 week after untreated rat chromaffin cells were transplanted near the lumbar spinal cord after CCI (Fig. 7, ▲), the mechanical hypersensitivity began to resolve, with near-normal sensitivity by 8 weeks, compared to CCI alone. At this time period, the range of raw latency scores in the ligated paw varied from 0.49 to 3.60 g, with 3.90 to 4.08 g in the nonligated paw. One week after treated chromaffin cells were transplanted near the lumbar spinal cord after CCI (Fig. 7, ▽), the mechanical sensitivity began to resolve, with near-normal absence of hypersensitivity by 8 weeks, compared to CCI alone. At this time period, the range of raw latency scores in the ligated paw varied from 0.50 to 3.60 g, with 3.90 to 4.08 g in the nonligated paw.

DISCUSSION

The initial report describing immortalization of chromaffin cells with tsTag argued that mitotic cells found

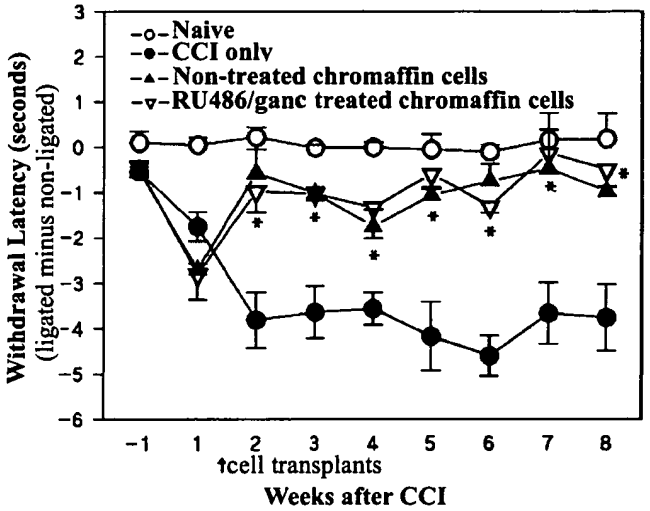

FIG. 6. Thermal hyperalgesia after CCI and chromaffin cell transplants. Animals were left unoperated (○), given the CCI (●), or transplanted with equal numbers of either untreated immortalized chromaffin cells (▲) or disimmortalized chromaffin cells (▽) 1 week following the CCI, 1 day following behavioral testing. Animals were tested for hindpaw withdrawal to heat once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated thermal hyperalgesia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate that both types of chromaffin cell transplants differed significantly from the CCI condition at each time point. $P < 0.01$.

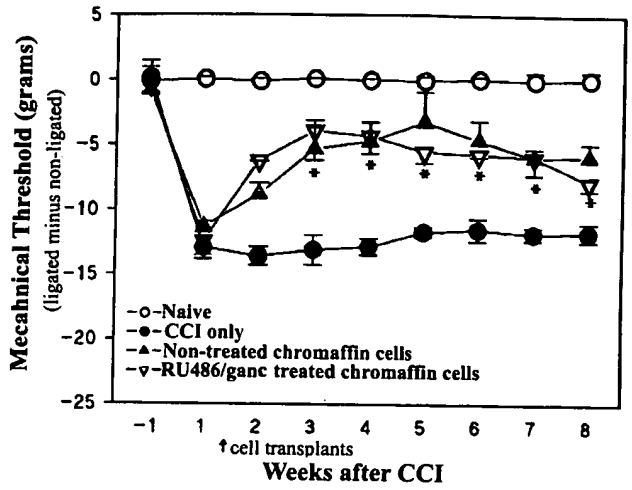

FIG. 7. Tactile allodynia after CCI and chromaffin cell transplants. Animals were either left unoperated (○), given the CCI (●) or transplanted with equal numbers of either untreated immortalized chromaffin cells (▲) or disimmortalized chromaffin cells (▽) 1 week following the CCI, 1 day following behavioral testing. Animals were tested for hindpaw withdrawal to a graded series of von Frey hairs once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated tactile allodynia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference scores for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate that both types of chromaffin cell transplants differed significantly from the CCI condition at each time point. $P < 0.01$.

in adrenal tissue could be conditionally immortalized with the temperature-sensitive Tag oncogene so that the differentiated cell type keeps many of the phenotypic features of primary chromaffin cells. Rat and bovine chromaffin cells immortalized in this manner express many of the catecholamine enzyme markers found in primary chromaffin cells and when differentiated *in vitro,* as the oncogenic Tag protein is degraded and mitosis ceases, these markers remained and were able to be regulated by continued differentiation, by agents such as dexamethasone and by stimulation of the cAMP pathway with forskolin. Such regulatory mechanisms are also seen in primary chromaffin cells. However, although these immortalized chromaffin cells are stable and appear homogeneous, catecholamine synthesis could not be detected by HPLC *in vitro* (15). But the currently described disimmortalizable rat chromaffin cells not only synthesize epinephrine after Tag excision, they also apparently make increased catecholamine enzymes, judged by qualitative immunohistochemistry for the enzymes compared to both nonexcised and those immortalized with only tsTag (15). Conferring immortalization with the SV40 large T antigen expression has a variety of effects on cells including inactivation of the growth suppressors pRB, p53, and SEN6 (33, 52), a decrease in G1 and increase in G2 and M cell cycle phase duration (55), and the ability of large T antigen to block the differentiation process (12, 26). However, after immortalization with the temperature-sensitive allele tsTag (23, 32), immortalized cells have been seen to resume the stage of life span and function of an uninfected cell when they are shifted to nonpermissive temperature conditions (31). These cells at the nonpermissive temperature have lost the ability to drive cell proliferation, since the large T antigen is labile at the higher temperature conditions (47), the T antigen is not able to drive mitosis in cells immortalized with the construct, and differentiation is favored (23, 32). In general, SV40 large T antigen-immortalized cell lines retain the phenotype of the differentiated lineage of the parent. A few functional neural cell lines have been immortalized with Tag and retain their specific catecholaminergic phenotype (44, 59) and efficacy to reverse neurological deficits after CNS transplant (13, 59). Cell lines of adrenal medullary origin have been established from SV40 Tag transgenic mice (7, 57), polyoma virus-induced tumors from postnatal mice (58); from a rat adrenal pheochromocytoma tumors (PC12 line (27)); and rat and bovine chromaffin cell lines from embryonic rat and neonatal calf, respectively (17). Even though these cell lines do not synthesize epinephrine *in vitro,* they may exhibit upregulation of the adrenergic phenotype after transplant *in vivo* (58), suggesting that adrenal medullary cell lines are subject to some forms of environmental regulation after transplant. When the ts allele is used as the transgene, the adrenal medullary cell lines derived from such mice retain the ability to respond to the nonpermissive temperature *in vitro,* shutting off proliferation, but they quickly die and are dependent on the Tag expression for viability and hence are not truly "conditionally immortalized" (7).

This study again demonstrates the ability (19) of immortalized (untreated) rat chromaffin cells to provide antinociception after partial nerve injury, and disimmortalized chromaffin grafts are also able to significantly reduce thermal and tactile hypersensitivity, when equal numbers of cells are placed in the subarachnoid space after CCI to the sciatic nerve. Rather than suggesting that antinociception is the result of catecholamine synthesis, release, or secretion, the existence of an equivalent functional effect by nondisimmortalized cells suggests that another agent or mechanism is responsible for reduction of neuropathic pain by these genetically manipulated chromaffin cells. We have suggested (19) that even if chromaffin grafts do not make significant levels of catecholamines *in vivo,* the antinociception the grafts provide might be a result of other antinociceptive molecules synthesized and released by the cells, such as GABA or Met-enkephalin. Presumably the increased norepinephrine phenotype recovered following excision of the oncogene by disimmortalized cells would function to advantage in cell therapy, but in the tests used in the present experiments no such advantageous effect could be demon-

strated. Rather, the value of disimmortalization before transplantation is to provide a measure of safety, with the complete absence of the oncogene and prevention of even a remote possibility of viral transfer of the large T antigen in the host, after grafting such cells.

Conditional immortalization allows for increased numbers of clinically useful therapeutic cell types that could be used in transplant paradigms (4, 6, 30, 37, 38, 43, 61). Lack of availability of primary cells for *ex vivo* gene therapy has often been the major barrier to the development of cell therapy for the reversal of degenerative diseases or traumatic injuries. However, creation of immortalized cell lines could not represent an alternative because of the appearance of a tumorigenic potential after immortalization. Moreover, immortalization with large T antigen has resulted in hyperdiploidaneuploid karyotype (34, 45, 62, 64), decreased differentiation (12, 26), and other chromosomal and cellular aberrations, depending on a minimum level of T-antigen expression in any cell that determines such interference with normal cell biology (45). Nevertheless, cell lines encoding temperature-sensitive large T antigen have been successfully used to modify host function and are able to differentiate in a variety of transplant paradigms without tumor production (8, 14, 18–22, 40, 41, 46, 53, 54, 63). These result show that shutting off the expression of the oncogene, as happens when cells immortalized by tsTag are grafted to animals that have a core temperature corresponding to a nonpermissive temperature for tsTag expression, can still lead to the expression of normal functional features. In this context it is interesting to note that the arrest of Tag expression following disimmortalization leads to an increase in the catecholamine-synthesizing enzymes and of catecholamine content in the chromaffin cells, possibly indicating an enhanced differentiation. Irreversible removal of a potentially subverting oncogene by its excision using the Cre/lox system might thus be a clinically useful strategy, especially since the core temperature of humans is lower than that of rodents, and the expression of a temperature-sensitive antigen might not be completely blocked in a clinical context (4, 6, 30, 37, 39, 61). Note that in this respect, use of modulatable Cre activity that can be activated by the synthetic steroid RU486 (35, 36) has added a means to select the timing of disimmortalization and renders the overall procedure more flexible and efficient. Unfortunately, as this study demonstrates, complete 100% disimmortalization is not yet accomplished, due to some sort of nonresponsiveness of a very small subpopulation of cells. It would be critical, if such methods were to be ever used clinically, for transplanted cells to be completely responsive to disimmortalization, for the sake of complete safety. However, we and others (unpublished observations) have observed that no Tag-containing cells survive transplant after pretransplant disimmortalization, but such a problem needs a vigorous examination. However, even with incomplete disimmortalization pretransplant, such technology offers the possibility, yet to be examined experimentally, to implant nondisimmortalized cells and proceed to the excision of the oncogene *in vivo* by treating the recipient with RU486 without the risk of having deleterious side effects following the administration of the drug. Indeed, with the use of CrePR1, the ligand-binding domain of the mutant progesterone receptor, hPR891HBD, confers RU486 responsiveness (35, 36, 60) *in vitro,* and *in vivo* at a dosage lower than that required to antagonize progesterone action, thus avoiding nonspecific effects of the drug, and such a system seems feasible for human use, even after transplants of immortalized cells are in place.

This report then represents a promising first attempt at reversible immortalization of chromaffin cells, demonstrating that disimmortalized chromaffin cells retain many features of the mature chromaffin cell phenotype. In summary, the availability of conditionally immortalized chromaffin cells for a variety of studies, including their use as transplants in various models of neuropathic and neurogenic pain, reflects the growing interest in the development of molecular biological techniques of cellular therapy for neurodegenerative diseases and CNS trauma. Such a renewable source will also allow for the manipulation of the chromaffin cell's genome to investigate the mechanisms of action responsible for cell grafts to repair the injured CNS environment. Similar immortalization of human chromaffin precursors and creation of human chromaffin lines presage the advent of cellular therapy as a therapeutic strategy for a variety of currently difficult clinical problems, including intractable pain after PNS and CNS trauma.

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